Ticking for Metabolic Health

Obesity

Ticking for Metabolic Health: The Skeletal-Muscle Clocks

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To be prepared for alternating metabolic demands occurring over the 24-hour day, the body preserves information on time in skeletal muscle, and in all cells, through a circadian-clock mechanism. Skeletal muscle can be considered the largest collection of peripheral clocks in the body, with a major contribution to whole-body energy metabolism. Comparison of circadian-clock gene expression between skeletal muscle of nocturnal rodents and diurnal humans reveals very common patterns based on rest/active cycles rather than light/dark cycles. Rodent studies in which the circadian clock is disrupted in skeletal muscle demonstrate impaired glucose handling and insulin resistance. Experimental circadian misalignment in humans modifies the skeletal-muscle clocks and leads to disturbed energy metabolism and insulin resistance. Preclinical studies have revealed that timing of exercise over the day can influence the beneficial effects of exercise on skeletal-muscle metabolism, and studies suggest similar applicability in humans. Current strategies to improve metabolic health (e.g., exercise) should be reinvestigated in their capability to modify the skeletal-muscle clocks by taking timing of the intervention into account.

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Study Importance

What is already known?

- Skeletal muscle is important in maintaining whole-body energy, substrate metabolism, and glucose homeostasis.
- Disruption of the skeletal-muscle clocks in mice leads to impaired glucose metabolism and increased insulin resistance.
- Circadian misalignment in humans results in disturbed energy metabolism and insulin resistance in skeletal muscle.

What does this review add?

- This is the first review focused on comparing the findings on skeletal-muscle clocks between humans and mice.
- We review the latest progress on the role of the skeletal-muscle clocks in metabolic health.
- We discuss time-dependent exercise as a therapeutic strategy for restoring metabolic disruption.

Introduction

Circadian rhythms are endogenously generated 24-hour cycles that can be observed in behavior, physiologic functions, and metabolic processes. The molecular mechanism that drives circadian rhythms is the circadian clock, and this is found in virtually every cell in mammals. Although the circadian clock has been studied most in the suprachiasmatic nucleus of the brain (central clock), we are now learning more about the role of the circadian clocks in peripheral tissues such as skeletal muscle. The molecular clock generates circadian rhythms through a translational-transcriptional feedback-loop (TTFL) mechanism, in which circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator–like 1 (BMAL1) proteins act as transcriptional activators of cryptochrome 1 and 2 (*Cry1* and *Cry2*) and period 1, 2, and 3 (*Per1*, *Per2*, and *Per3*) genes, which encode proteins that repress CLOCK:BMAL1 with a periodicity of ~24 hours (Figure 1). In addition to the TTFL, CLOCK:BMAL1 also contributes to the expression of a large number of genes, called clock-controlled genes (CCGs). Transcriptomics has been the most used tool to identify CCGs in mammalian tissues. Early skeletal-muscle transcriptomic studies relied on sampling every 4 hours and identified >200 rhythmic genes in mice, including components of the molecular clock and genes involved in transcription, lipid metabolism, protein degradation, ion transport, and vesicular trafficking (1,2). Zhang et al. (3) performed a circadian time-series analysis of gene expression in mice every 2 hours for 48 hours in 12 different mouse organs, including skeletal muscle (gastrocnemius), using microarrays and targeted RNA-sequencing data. They found that skeletal muscle expresses ~1,600 circadian genes and that more than 40% of all mammalian protein-coding genes are expressed rhythmically somewhere in the body, mainly in an organ-specific manner (3,4). The components of the core circadian-clock mechanism are ubiquitous across all cells; however, the CCGs are unique to each tissue. Skeletal muscle–specific CCGs include myogenic differentiation 1 gene (*Myod1*), *Atrogin1* gene (Fbxo32), myocyte enhancer factor 2 gene and glucose transporter type 4 gene (*Glut4*), highlighting the role of the clock for expression of genes important for the daily maintenance of skeletal muscle (1,4).

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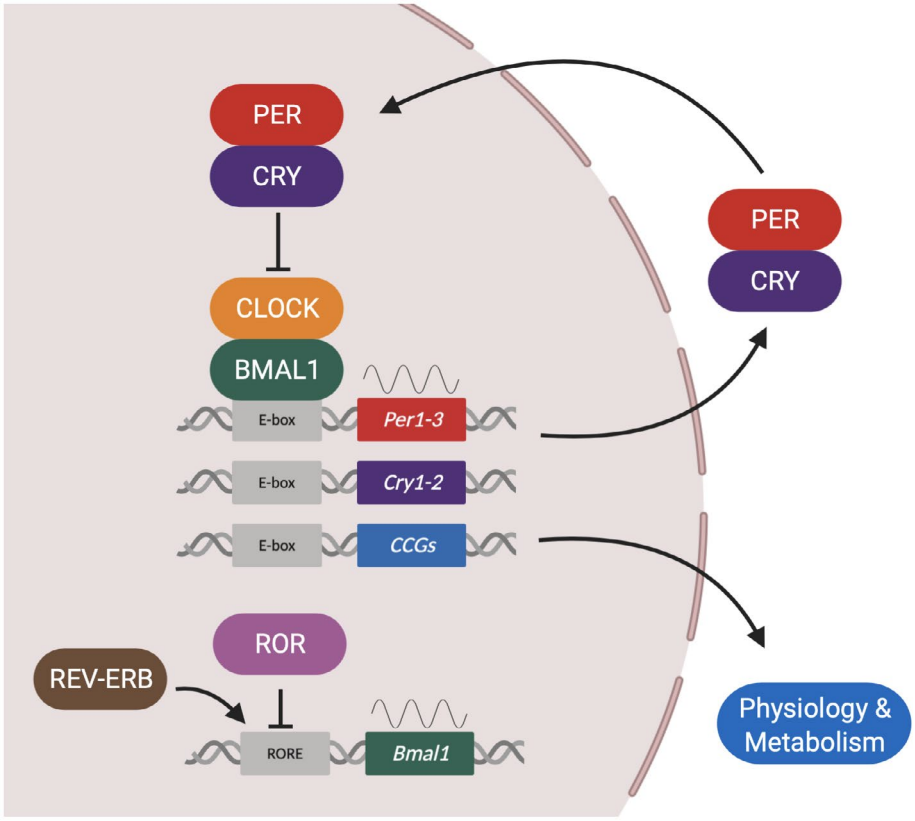

Figure 1 The molecular clock generates circadian rhythms through a transcriptional-translational feedback loop (TTFL) mechanism, in which the transcription of Period (*Per1*, *Per2*, and *Per3*) and Cryptochrome (*Cry1* and *Cry2*) genes is activated by the complex formed by the PAS domain helix-loop-helix transcription factors CLOCK (or its paralogue NPAS2) and *BMAL1* (also known as ARNTL) via E-boxes elements in their promoter regions. PER and CRY proteins dimerize, translocate to the nucleus, and repress their own CLOCK:*BMAL1*-mediated transcription. This creates a cycle that takes approximately 24 hours to be completed. In addition to the main loop, a secondary feedback loop comprises the nuclear receptors ROR and REV-ERB that activates or represses, respectively, the expression of Bmal1 gene by acting on ROR-binding elements (ROREs) within its promoter. CLOCK:*BMAL1* activates the transcription of clock-controlled genes (CCGs) in a tissue-specific manner and these genes are critical for cell physiology and metabolism.

By constituting ~40% or more of body mass, skeletal muscles can be considered the largest collection of peripheral clocks in the human body (5). In the context of metabolic health, the relevance of studying skeletal-muscle clocks in particular becomes clear, considering the important role of skeletal muscle in maintaining whole-body energy, substrate metabolism, and glucose homeostasis; one example being that skeletal muscle accounts for ~80% of postprandial glucose uptake in humans (6,7). Skeletal muscle is also a metabolically active tissue, especially when considering the energy cost of skeletal-muscle force, as well as the pumps managing intracellular calcium stores and those pumps maintaining membrane potential, thereby being the largest contributor to the whole-body energy requirement (8-10). Moreover, skeletal muscle is able to rapidly adapt to metabolic demand (e.g., exercise) and nutrient status by modulating substrate storage and matching substrate oxidation to glucose or fat availability, respectively (11). Although its flexibility to either use or store energy is mostly dependent on the change from feeding to fasting and from activity to rest, recent evidence suggests that skeletal-muscle metabolism is further governed by the intrinsic skeletal-muscle clocks (4).

This review summarizes the literature on how the skeletal-muscle clocks of nocturnal rodents and diurnal humans are organized. The ability to perform repeated biopsies of skeletal muscle from the same individual over a 24-hour period provides an opportunity to directly compare the expression of circadian-clock factors between humans and rodents. On the basis of these comparisons, we discuss the relevance of findings from clock-disruption rodent models for human metabolic health. The aim is to evaluate the physiologic relevance of the skeletal-muscle clocks for human metabolic health and to give an outlook on how future studies could usefully further investigate clockwork in skeletal muscle.

Study of Circadian Rhythms in Nocturnal Rodents and Diurnal Humans

The proper functioning of the circadian clock in skeletal muscle is likely to be crucial for the regulation of whole-body energy and substrate metabolism. However, scientific interest in skeletal-muscle clocks in

humans has only recently emerged, so that our current knowledge about skeletal-muscle clocks is mostly based on studies in nocturnal rodents. Evolutionarily diverged from humans approximately 96 million years ago (12), the mouse is widely viewed as a representative model to study mammalian gene expression. However, particularly when it comes to investigating circadian rhythms, differences from humans should be considered. Among the obvious differences are the opposing times of activity and feeding versus rest. For example, in preclinical studies, mice are generally fed ad libitum, but data indicate that 75% to 80% of intake occurs in the dark phase (13,14). Mice also exhibit polyphasic or segmented bouts of sleep, but the majority of sleep episodes are in the light phase (15). In contrast, humans are physically active and eat mostly during the light phase and exhibit consolidated sleep in the dark phase. Thus, most of the rhythms found in behavior, physiologic function, and metabolism are antiphase when comparing nocturnal and diurnal animals against the light/dark (LD) phases of the day.

The analysis of circadian rhythms requires standardized conditions to control for environmental cues. Under normal circumstances, mice are housed under defined conditions with regard to lighting, feeding, and activity, and tissues can be collected at any given time of the day to investigate rhythmic patterns in skeletal muscle and other tissues. The current guidelines for discovering circadian patterns recommend that tissues be collected for at least 2 complete 24-hour cycles, during which mice are under constant dark conditions with no input from light (16).

For human studies, consistent conditions (e.g., regular meals, physical activity, and bedtimes) are critical. However, to study the human skeletal-muscle clocks, a more elaborate and careful study design is required to avoid confounding factors in the face of ethical standards. For this purpose, two approaches have currently been used: constant-routine or realistic-lifestyle protocols. Constant-routine protocols are designed to avoid confounding factors, known to affect skeletal-muscle clocks, as much as possible. For example, Perrin et al. (17) collected skeletal-muscle biopsy specimens every 4 hours over 24 hours in 10 healthy volunteers constantly in a semirecumbent position, under constant hourly feeding via meal-replacement solutions and constant artificial lighting and ambient temperature. In contrast, van Moorsel et al. (18) collected 5 skeletal-muscle biopsy specimens within 24 hours from 12 young healthy male participants under "normal" living conditions (i.e., 3 meals per day, some physical activity) to mimic realistic daily lifestyle conditions as much as possible. Sleeping was facilitated for 8 to 9 hours with lights switched off in both protocols. The direct comparison of specific analyses, such as metabolome or transcriptome analyses, in muscle samples derived from these two different protocols may contribute to a better understanding of the relative roles of the circadian versus behavioral components of biological rhythmicity.

The Skeletal-Muscle Clocks in Rodents and Humans

Circadian-clock genes have been shown to exhibit 24-hour rhythmic expression in human and mouse skeletal-muscle samples. In this section, we will refer to *LD*, but we will also include the descriptors *rest*/*active* as a way to compare the daily patterns of clock gene expression between rodents and humans. The core clock genes *Bmal1* and *Clock* function as the positive arm of the TTFL mechanism, and in mouse skeletal muscle, *Bmal1* mRNA exhibits a peak expression at the transition from the dark/active to the light/rest phase. In contrast, the genes that make up the negative arm of the clock, *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2*, exhibit an antiphase pattern with their peak of expression prior to the transition from rest to the active phase (3,4). Expression of core clock genes have also been measured in the human skeletal muscle of lean volunteers, both when measured directly in human skeletal-muscle biopsy specimens (17-19) and by *in vitro* differentiation of human primary skeletal myotubes established from human-donor biopsy specimens (17,20,21). *In vivo* studies have shown that *Bmal1* expression also peaks during the transition from the active to the rest phase in human skeletal muscle, whereas *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2* are antiphase to *Bmal1*, and their expression peaks at the transition from rest to the activity phase. The expression of *Clock* mRNA is more variable with either very low amplitude (17,18) or no circadian variation (21). Expression patterns of the clock genes *in vitro* are less robust. In differentiated human myotubes, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2* are rhythmic after synchronization with forskolin (17). A previous study found oscillations in *Bmal1*, *Per2*, *Per3*, and *Cry1* after synchronization with dexamethasone (20). Hansen et al. (21) reported rhythmic expression in the same genes, but *Per1* and *Cry2* could not be detected, after serum shock synchronization. In differentiated myotubes of C2C12s, a mouse skeletal-muscle cell line, the expression of *Clock*, *Bmal1*, *Cry1*, *Per1*, and *Per2* was found to be rhythmic after dexamethasone synchronization (22). This direct juxtaposition of available data on the skeletal-muscle clocks in mice and humans reveals that if expression is normalized to activity/rest cycles, the skeletal-muscle clocks of humans and mice show very common patterns of gene expression, as illustrated in Figures 2A-2B.

Beyond the core clock factors, Perrin and colleagues (17) performed a genome-wide transcriptome analysis by high-throughput RNA sequencing from skeletal-muscle biopsy specimens from 10 healthy volunteers. A comparison of the oscillating genes found by Perrin et al. (17) with the circadian gene-expression data set of mouse skeletal muscle previously published by Zhang et al. (3) revealed 430 common circadian oscillating genes between mouse and human skeletal muscle. Surprisingly, the phase difference in the expression of the core clock components between diurnal humans and nocturnal rodents was shorter than the expected 12-hour phase difference. This likely reflects the differences in entrainment, as humans were entrained to a 15-hour/9-hour LD cycle, whereas mice were kept on a 12-hour/12-hour LD cycle prior to being placed in constant darkness during tissue collection. It is also important to note that the mouse data set had a temporal resolution of 2 hours, whereas the human temporal resolution was 4 hours, and this could have impacted phase determination (3,17). It also needs to be taken into account that human studies are primarily done with biopsy specimens from the vastus lateralis, whereas most mouse studies investigate muscles of the lower leg, such as the gastrocnemius muscle. So far, there are no data sets for direct skeletal-muscle-type comparisons between humans and mice.

To date, transcriptomic studies have been published on three different mouse skeletal muscles. Dyar et al. (23) evaluated the expression of circadian genes in two skeletal muscles with distinct fiber-type composition: the soleus, which is composed of a mixed type 1/type 2 fiber and has a majority of oxidative fibers (24), and the tibialis anterior (TA), composed of mostly fast-twitch glycolytic fibers (24). They identified 1,359 circadian genes in the soleus, whereas 684 circadian genes were found in the TA. Interestingly, a majority of CCGs were specific to each skeletal muscle, with 75% and 51% of circadian genes cycling only in the soleus or TA, respectively (25). Circadian transcriptome

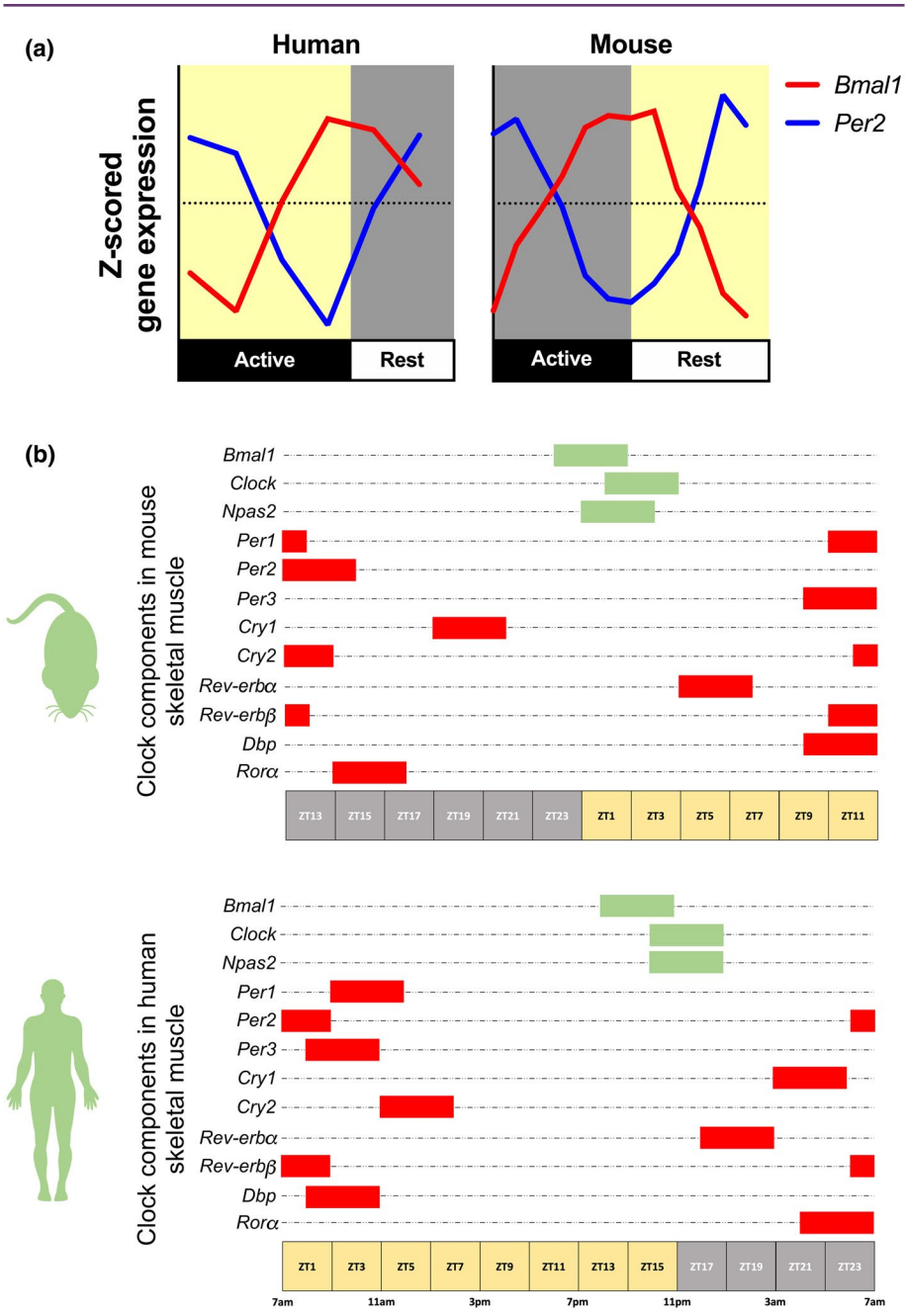

Figure 2 (**A**) Normalized expression of core clock genes, brain and muscle aryl hydrocarbon receptor nuclear translocator–like 1 (*Bmal1*) and period 2 (*Per2*), in skeletal muscle of humans and mice over time of day. The patterns of *Bmal1* and *Per2* expression are similar when viewed from the rest and active phases of the day. Human skeletal-muscle data were extracted from Perrin et al. (17), and mouse gastrocnemius data were extracted from Zhang et al. (3). The *Bmal1* circadian-expression profile is represented by the red line, and the *Per2* circadian-expression profile is represented by the blue line. (**B**) Representation of peak times of expression of a larger composite of clock genes and clock-controlled genes (CCGs) in mouse skeletal muscle (top panel) and human skeletal muscle (bottom panel). Genes of the positive limb are represented by green bars, and genes of the negative limb are represented by red bars. Zeitgeber time 0 (ZT0) refers to the time when lights are switched on (e.g., at 7:00 AM in human studies). For illustration purposes, 3-hour time frames were used, taking the mean value of peak expression of the respective gene from comparable human (17,18) and mouse (3,4,23) studies as the center. Note that if corrected for the rest/activity cycle, peak expression patterns across the clock components are similar in both species. *Clock*, circadian locomotor output cycles kaput gene; *Cry*, cryptochrome gene; *Dbp*, D site of albumin promoter binding gene; *Npas2*, neuronal period/agouti-related transcript/single-minded domain 2 gene; *Rorα*, retinoic acid receptor–related orphan receptor alpha gene.

studies using the mouse gastrocnemius muscle (~75% type 2 glycolytic, ~12% type 2 oxidative, and ~13% type 1 oxidative fibers) found diverse signatures, with greater than 50% of the genes being differentially expressed (1-4). Thus, although all the different skeletal muscles express the core circadian clock factors, the differences in CCGs across skeletal muscles highlights the specificity of circadian regulation and may reflect differences in skeletal muscle use, metabolism, and/or functional properties. In addition, it is likely that there will be sex-specific differences; however, to date, there are few data on sex as a variable for the circadian-clock mechanism in skeletal muscle.

Among the molecular pathways held in common between human and mouse circadian data sets is the category of gene transcription. As previously demonstrated in mice, gene-transcript accumulation followed a rhythmic pattern, with peaks in the middle of both the active and rest phases in humans (17). The active-phase peak was characterized by enhanced expression of genes associated with mitochondrial activity and skeletal-muscle contraction. In contrast, the homologous genes in rodents were partitioned across both the active and rest periods (1,2,4). In humans, the genes peaking during the middle of the rest period were enriched for the immune response and inflammation pathways. Furthermore, various transcription factors related to skeletal-muscle metabolism demonstrated a rhythmic expression, such as Krueppel-like factor 15 gene, which plays a crucial role in regulating skeletal muscle lipid metabolism (26); transcription factor EB gene, which regulates oxidative phosphorylation and glucose homeostasis (27); and *Myod1*, the master myogenic regulatory factor, which peaks during the active phase. *Myod1* was previously identified as having a robust circadian pattern of expression in mice, with peak expression during the active phase (2). Since this early observation, studies have demonstrated that *Myod1* is a direct target of the CLOCK:BMAL1 complex (28) and have identified it as a clock cofactor regulating the expression of CCGs in skeletal muscle (29,30). It will be interesting to determine whether this role for *Myod1* is conserved between mouse and human skeletal muscle. In addition to the transcription factors, genes involved in the secretion of myokines, glucose homeostasis, and lipid metabolism displayed rhythmic transcription in humans (17). Taken together, these transcriptomic data strongly suggest an important role of the skeletal-muscle clocks in regulating human and mouse muscle physiologic function.

Indeed, skeletal-muscle clocks may contribute to the circadian rhythms of human resting energy expenditure, with the lowest levels seen in the late biological night and the highest levels seen in the afternoon/evening. Another metabolic variable, respiratory exchange ratio (RER; carbon dioxide production/oxygen consumption), exhibits a circadian pattern indicating a preference for carbohydrate oxidation in the biological morning and lipid oxidation in the evening, as was reported recently (31). Furthermore, exercise performance displays diurnal variation: for example, increased strength, power, and endurance in the afternoon and evening compared with the early morning (32,33). In addition, glucose tolerance and insulin sensitivity were shown to be higher in the morning compared with later on in the day in healthy individuals (34). Further, we showed pronounced 24-hour rhythmicity of skeletal-muscle mitochondrial function in humans, with a peak during the late phase and a trough during the early phase of the day, both for basal and maximally-stimulated mitochondrial respiration (18). However, with the "normal" lifestyle protocols applied in these examples, the rhythms in these physiologic markers cannot only be attributed to the influence of the skeletal-muscle clocks, per se, but can also be attributed to external factors, such as the nutrient composition of the provided meals (i.e., the higher fat content of dinner compared with breakfast and lunch), as well as the timing of the meal relative to the biopsy specimen taken (although biopsy specimens were always taken just before each meal to avoid direct postprandial effects).

Disruption of Skeletal-Muscle Clocks and Its Effects on Metabolism

Chronic disruption of circadian rhythms has been associated with metabolic disturbances in humans and mice (35). The importance of skeletal-muscle clocks in physiologic function and behavior has been addressed using genetic loss-of-function mouse models targeting *Bmal1*. *Bmal1* is the common target, as it is the one nonredundant component of the clock mechanism. Global *Bmal1* knockout (KO) mice are characterized by an arrhythmic behavior under free-running conditions, growth retardation at 16 to 18 weeks of age, and a very short lifespan (average of 37 weeks), with hallmarks associated with premature aging, such as skeletal-muscle wasting, cataracts, hair loss, arthropathy, and age-related body and organ reduction (36,37). Metabolically, these mice show no circadian variation in plasma glucose or triglycerides, a profound hypoglycemic response after insulin administration, and an impaired conversion of exogenous pyruvate to glucose (38). The size of skeletal muscle in the global *Bmal1* KO mice was first described to be reduced in an age-dependent manner (37). A more detailed analysis of the skeletal-muscle cell structure and function showed that the global *Bmal1* KO mice have a reduction in force generation, a significant myofilament and sarcomere disorganization, and mitochondrial pathologic conditions, highlighting the importance of the skeletal-muscle clocks in the maintenance of skeletal-muscle function and metabolism (28).

Skeletal muscle–specific *Bmal1* KO mouse models have been generated to study the effects of circadian disruption on skeletal muscle. While there are some differences in strength outcomes, these mouse models have a common phenotype that includes disrupted regulation of insulin-stimulated glucose uptake and its metabolism (23,39). Skeletal muscle–specific *Bmal1* KOs exhibited reductions in the mRNA and protein levels of GLUT4 and TBC1 domain family member 1. In addition to the change in glucose transport, the decrease in *Bmal1* in skeletal muscle causes a downregulation of the key enzymes for the use of glucose, such as hexokinase 2 and pyruvate dehydrogenase (4,23). The skeletal muscle–specific *Bmal1* KO mice display normal circadian behavior under free-running conditions, unlike the global *Bmal1* KO mice, suggesting that the changes observed in the metabolism are more likely to be downstream of the clock mechanism in skeletal muscle. These results indicate that the circadian clock generates the circadian rhythm of carbohydrate metabolism in skeletal muscle and that its disruption leads to metabolic dysfunction in skeletal muscle.

Complementary to the data from the *Bmal1* KO mice, circadian transcriptomic studies have been performed using the *Clock*-mutant mouse. It is important to note that this is neither a KO nor a knockdown mouse, but it is a mouse that carries a nonspecific skeletal-muscle mutation in the *Clock* gene that leads to a dominant negative-type version of CLOCK in the whole mouse (40,41). These studies (1,2) found that 78% of the rhythmic genes in wild-type skeletal muscle had an altered magnitude of expression levels in *Clock*-mutant skeletal muscle, whereas 11% of the rhythmic genes from wild-type skeletal muscle were shifted out of phase in the *Clock*-mutant mice. Analysis of these data sets revealed that CLOCK-regulated genes were associated with the cell cycle and cell proliferation, the insulin

signaling pathway, protein translation, gluconeogenesis, and muscle contraction (1).

The silencing of core clock genes and its effect on metabolism have also been evaluated *in vitro* using human skeletal muscle cells. Perrin et al. (17) cultured primary skeletal myotubes and disrupted their circadian clock by transfecting small interfering RNA targeting *Clock* (si*Clock*). Genes involved in contraction-induced and insulin-stimulated glucose uptake were downregulated on si*Clock*. Accordingly, basal and insulin-stimulated glucose uptake in these myotubes was markedly reduced. Taken together, these findings suggest that, like *Bmal1*, the *Clock* gene/protein in skeletal muscle is important for coordinating glucose uptake *in vitro*. Moreover, *Clock* depletion induced changes in lipid homeostasis (17) and myokine secretion (20). As shown in the skeletal muscle–specific *Bmal1* KO mouse model, *Clock* disruption also facilitated a global switch at the transcriptomic program toward a more oxidative program (4,17). Various genes involved in lipid transport and storage were affected after *Clock* silencing, leading to alterations in total phosphatidylcholine and glycosylceramide levels. In particular, an increase in the long-chain fatty acid transporter CD36 and an increase in fatty acid binding protein 3, consistent with the mouse skeletal-muscle clock-disruption model, was found on *Clock* silencing (4).

Loizides-Mangold et al. (19) performed a targeted lipidomic analysis to quantify circadian rhythmicity within different lipid classes in human skeletal-muscle samples. In line with findings from lipidomic analysis of human plasma (42), ~20% of detected lipids displayed diurnal oscillation *in vivo* and *in vitro*. Most lipids peaked in the early morning phase prior to awakening. As lipids largely contribute to the formation of the plasma membrane, it can be assumed that skeletal-muscle cell-membrane properties are subjected to substantial changes over the active/rest cycles, potentially dictating important metabolic processes (e.g., receptor signaling and glucose uptake). Moreover, peak levels of major glycerophospholipids and sphingolipids were found to correlate with the peak of *Bmal1* expression in both *in vivo* and *in vitro* conditions (19), indicating a potential role for skeletal-muscle clocks in the regulation of lipid metabolism. Importantly, ~40% of oscillating lipid metabolites were downregulated in myotube cultures after si*Clock* treatment. Furthermore, the analysis of lipidomic data indicated that there were also changes in the period length and amplitude of the oscillations after si*Clock* treatment (19). It is not clear what the impact of period-length changes will be for skeletal muscle, but it highlights the variety of possibilities regarding how the circadian clock can impact metabolic outcomes. Mouse skeletal-muscle transcriptomics have demonstrated that genes involved in fatty acid uptake and β-oxidation peak in the middle of the rest phase, whereas lipogenic genes reach peak expression at the end of the active phase, suggesting that the clock in skeletal muscle promotes storage of excess energy at the end of the active phase (4).

Circadian Misalignment and Its Impact on Skeletal-Muscle Metabolism

Circadian misalignment is often used in both mouse and human studies to explore the consequences of circadian disruption in an experimental setting. Circadian misalignment is defined by disruption of clock phases across the tissues/organs within the system. This occurs through disturbing normal circadian time cues relative to the behavioral cycle, including altered phases of LD, feeding/fasting, and activity/rest. In modern society, many forms of circadian misalignment already exist, such as common daily routines like artificial lighting at night, eating at night, and sleep deprivation, but this misalignment is also caused by working conditions (e.g., shift work and time-zone transitions resulting in jet lag). Although there are many different ways to induce circadian misalignment, it is unclear if all these forms of misalignment lead to similar effects on parameters of metabolic health. However, a recent meta-analysis of observational studies revealed that shift workers, who are exposed to a mixture of disrupted light exposure, eating at night, and sleep deprivation, have an increased risk of developing type 2 diabetes mellitus (T2DM) (43), and the risk is positively correlated with the number of night shifts per month (44). To study the underlying mechanisms of this relationship, circadian misalignment protocols have been developed to simulate conditions of shift work by shifting the behavioral cycle (including the sleep/wake and fasting/feeding cycles).

Wefers and colleagues (45) tested 14 healthy lean volunteers in a randomized crossover design for 3.5 days of either shifting the behavioral cycle by 12 hours or a control condition. At the end of both periods, insulin sensitivity was determined using the gold-standard hyperinsulinemic-euglycemic clamp at the beginning of the active phase for both control and misaligned protocols, and skeletal-muscle biopsy specimens were taken for mRNA analysis. Wefers et al. (45) found that the circadian rhythm of core body temperature did not adapt to the new day/night rhythm (i.e., indicating circadian misalignment), leading to a higher body temperature and energy metabolism during sleep and a reduced body temperature during the waking period in the misaligned condition. Interestingly, this circadian misalignment resulted in an increase in plasma glucose and free fatty acid levels and a rapid induction of insulin resistance, which was mainly located at the level of the skeletal muscle, as indicated by a 23% reduction in insulin-stimulated nonoxidative glucose disposal. Whole-genome expression profiling revealed that many of the most highly enriched gene sets among the genes changed in expression in the misaligned condition were related to fatty acid metabolism and peroxisome proliferator–activated receptor signaling (45). In addition, the expression of clock genes at two time points, 7:00 AM and 7:00 PM, was determined in both conditions. In the control condition, the core clock genes *Bmal1*, *Cry1*, and *Per2* displayed diurnal differences in line with those of the previously described studies (17,18). Despite reversing the activity/rest and feeding/fasting cycle, the expression pattern of *Bmal1* and *Clock* was conserved, highlighting the robustness of the positive limb of the skeletal-muscle clocks to feeding and activity, whereas the expression of *Per2* and *Cry1* mRNA was altered.

Another study investigated the effects of acute sleep loss compared with a full night of sleep on the transcriptome of skeletal muscle and adipose tissue, including their clock machinery (46). Strikingly, after only one night of sleep loss, pathways indicative of skeletal-muscle breakdown and anabolic processes in adipose tissue were evident. At the same time, only the skeletal-muscle clocks were altered, with an upregulation of BMAL1 protein levels, whereas the molecular clock in adipose tissue remained unchanged. Interestingly, sleep deprivation also resulted in molecular changes in skeletal muscle, indicating a shift to fatty acid metabolism, supporting the findings by Wefers et al. (45). Sleep loss further led to higher postprandial glucose levels, whereas insulin was stable, suggesting reduced glucose handling in peripheral tissues (46). Accordingly, it has been shown that persistent sleep restriction with concurrent circadian disruption alters metabolism and could increase the risk of obesity and T2DM (47). Taken

together, these data indicate that circadian misalignment results in disturbed energy metabolism and insulin resistance in human skeletal muscle, which both could be mediated by impaired skeletal-muscle clocks.

The Skeletal-Muscle Clocks in Obesity and Under Diabetogenic Conditions

The circadian metabolic profile of skeletal-muscle cells has further been evaluated under diabetogenic conditions *in vitro*. Hansen and colleagues (21) compared clock gene expression of human primary myotubes derived from four different groups of donors: (1) young endurance-trained athletes; (2) their young, age-matched, lean sedentary controls; (3) healthy participants with obesity; and (4) BMI- and age-matched patients with T2DM. They did not find any differences in the expression of the core clock components between donor groups. Likewise, Perrin et al. (20) did not find any differences in the period length of the *Bmal1* or *Per2* luciferase (luc) reporters between myotubes established from donors with and without obesity. However, patients with T2DM lacked robust circadian rhythmicity of components of the core clock, *Rev-erbα* and *Rev-erbβ* (21). Downstream of the core clock, circadian rhythmicity in the expression of nicotinamide phosphoribosyltransferase gene (*Nampt*), sirtuin 1 gene, and insulin receptor substrate 1 gene, genes associated with mitochondrial function and metabolic health, were disrupted in myotubes from participants with T2DM (21). *Nampt* has been shown to be regulated by *Bmal1* and *Clock* in complex with sirtuin 1 gene (48), and sleep loss seems to affect rhythmicity of *Nampt* expression and might thereby contribute to impaired postprandial glucose metabolism (49). These results show that primary skeletal-muscles cells were able to maintain the circadian metabolic characteristics *in vitro*, depending on the donor characteristics, resulting in an advantageous *in vitro* tool to study the circadian clock from skeletal-muscle cells derived from patients with distinct metabolic diseases.

Recently, Sardon Puig and colleagues (50) assessed clock gene expression in the vastus lateralis muscle before and 6 months after gastric bypass surgery in women and men with obesity and compared these data to baseline gene expression in controls with normal weight. They found that skeletal-muscle-clock gene expression is affected by obesity and by gastric bypass–induced weight loss. However, these results must be interpreted with caution, as only one biopsy specimen was taken at each occasion, and the time of day was not standardized for the biopsy specimens taken at baseline. Accordingly, reported differences can be a result of timing differences rather than reflecting weight loss– or obesity-induced effects. In the same study, unsynchronized myotubes from the controls with normal weight were exposed to plasma free fatty acids (i.e., palmitate or oleate) to simulate diabetogenic conditions *in vitro* and study the plasticity of the skeletal-muscle clocks under these conditions. For both fatty acids, the expression of a ubiquitous CCG, D site of albumin promoter binding gene (Dbp), was reduced, whereas *Rev-erbβ* was upregulated. In addition, serum shock-synchronized myotubes were exposed to palmitate for 54 hours. Over time, palmitate treatment was found to alter the expression of *Bmal1*, *Cry2*, D site of albumin promoter binding gene, *Per1*, and *Per3*. These findings suggest that circulating lipid metabolites can modify the phase and/or pattern of skeletal-muscle-clock gene expression. It could therefore be speculated that increased lipid signaling represents a putative component of the time-of-feeding cue for skeletal muscle, considering that free fatty acids rise during the fasted night under metabolically healthy conditions in humans (18). As free fatty acids are generally elevated in patients with T2DM (51), diabetic skeletal muscle might lose its sense of time based on nutrient status and may be further confronted with contradicting time cues during the daytime.

Exercise as a Tool to Reset the Clock and Improve Metabolic Health?

It is well known that exercise training is an effective strategy for restoring metabolic outcomes in the skeletal muscle of patients with T2DM to levels of age- and BMI-matched healthy controls (e.g., mitochondrial function, RER, and skeletal-muscle insulin sensitivity) by improving skeletal-muscle function (52). Whether exercise exerts its benefits through the molecular-clock mechanism has not been studied yet. However, acute exercise is now considered a time cue for clocks in peripheral tissues and joins other factors, such as glucocorticoids, feeding, neurohumoral input from the central clock, and (likely) temperature (53,54). Many studies using the circadian reporter *Per2::Luc* in mice have shown that running exercise can produce a significant time-dependent phase shift of the *Per2::Luc* bioluminescence rhythms in several skeletal muscles. It has also been shown that exercise can improve circadian rhythms in a genetic mouse model that uncouples the temporal communication within the central clock and therefore exhibits diminished behavioral and physiologic rhythms (55). Schroeder et al. (55) found that allowing these mutant mice to run on a wheel in the later part of the active/dark phase improved their rhythmic deficits, including an improved power of locomotion-activity rhythms, a shifting in the acrophase of locomotion-activity rhythms such that the phase was no longer different when compared with wild-type mice, and an increased amplitude of *Per2::Luc* rhythms in the central clock. Interestingly, the phase of *Per2::Luc* rhythms in peripheral tissues (e.g., the adrenals and liver) was also rescued.

In humans, data on acute-exercise and exercise-training effects on the skeletal-muscle clocks are more limited. Zambon et al. (56) compared skeletal-muscle-clock gene expression in the exercised and nonexercized leg of 4 human volunteers 6 hours and 18 hours after an acute bout of resistance exercise. First of all, they could confirm diurnal differences in the core clock factors within the nonexercized control limb. Strikingly, they found that *Bmal1*, *Per2*, and *Cry1* were upregulated 6 hours after exercise, whereas *Per1*, *Cry2*, and *Rev-erbβ* remained unchanged. This suggests that skeletal muscle contraction maybe sufficient to alter the gene expression of skeletal-muscle clocks, but the effect of exercise on physiologic whole-body or muscle-specific circadian outcomes was not investigated. Youngstedt et al. (57) investigated human circadian-phase response curves of urinary melatonin profiles after different times of moderate-intensity walking exercise performed for 1 h/d for 3 days. Based on a large sample size and under highly standardized conditions, exercise induced consistent phase advances when performed either at 7:00 AM or between 1:00 and 4:00 PM, and consistent phase delays were experienced when exercise was performed at 10:00 PM. Future studies are needed to determine if such phase advances or delays also occur within the skeletal-muscle clock. Previous studies investigating the effect of repeated daytime endurance exercise suggested a phase advance on the basis of dim-light melatonin onset (58) and rectal temperature (59). In contrast, acute nocturnal endurance exercise (for 3 hours) induced a phase delay in melatonin secretion(60,61).

There have been several recent studies investigating the time-dependent effects of exercise on metabolism. One recent study showed that

the time of acute exercise (early rest phase vs. early active phase) modifies the diurnal rhythmicity of the transcriptome in mouse skeletal muscle in a time-of-day–dependent manner (62). A combined analysis of transcriptomics and metabolomics in skeletal muscle showed that exercise during the early active/dark phase disrupts circadian rhythmicity of genes and metabolites related to carbohydrate metabolism, whereas exercise during the early rest/light phase stimulates the circadian expression of genes and metabolites related to carbohydrate metabolism and decreases rhythmicity of genes and metabolites related to glycerol metabolism (62). Moreover, the impact of acute exercise on systemic energy homeostasis appears to be dependent on the time of day, as oscillations in RER and energy expenditure respond differently according to the timing of exercise (62). Another study examined the difference in the exercise capacity of mice between 2 time points within their active phase, 2 hours after lights-off and 2 hours before lights-on (named *Early* and *Late*, respectively) (63). First, they reported that running duration at 55% and 45% of the maximal aerobic capacity was longer at the Late time. Gene expression analysis of skeletal muscle samples of nonexercized mice at Early and Late time points showed daily variation in exercise-related signaling pathways, including peroxisome proliferator–activated receptor, adenosine monophosphate–activated protein kinase, and hypoxia-inducible factor (63), suggesting that circadian regulation of these pathways may explain daily variation in exercise capacity, which has also been reported previously (62,64-66).

So far, human studies investigating the effect of exercise timing on the outcomes of training are very limited. However, in line with the above-mentioned findings in acute-exercise studies, Savikj et al. (67) used a randomized crossover design to compare outcomes for participants with T2DM who trained in the morning (1 hour after a meal) with those of participants with T2DM who trained in the afternoon (3 hours after a meal). They found that 2 weeks of exercise training when performed in the afternoon is more efficacious than morning exercise at improving 24-hour blood glucose levels in patients with T2DM. Ezagouri et al. (63) had human volunteers exercise using a submaximal constant-load exercise protocol at 2 times points, 8:00 AM (Early) and 6:00 PM (Late). The results confirmed a diurnal variance in exercise capacity, in which the Late group (vs. Early) was characterized by lower oxygen consumption, higher RER, lower heart rate, and lower blood glucose levels after exercise. To what extent the skeletal-muscle clocks might contribute to improved glucose tolerance in response to different timing of exercise training still needs to be elucidated, as, for example, the timing of meals may have also influenced these results. Nevertheless, these studies provide a rationale to continue to test the application of exercise timing as a therapeutic strategy to treat metabolic diseases in which the skeletal-muscle clocks might be disrupted.

Conclusion

Nowadays, in a mostly unnoticed manner, circadian disruption is omnipresent. In our modern society, especially, food and artificial lighting are available around the clock. These environmental cues are coming at irregular times, potentially providing conflicting time cues for all of the clocks in our body. With physical-activity levels declining, the contribution of exercise as a time cue for our clocks is underrepresented. Skeletal muscle is an important organ for metabolic health, and animal and human data reveal the relevance of circadian rhythmicity for skeletal-muscle metabolic health. The common features of the skeletal-muscle clocks in rodents and humans and the similar impact of clock disruption on substrate metabolism are important. An implication of these similarities is that preclinical research and clinical interventions can work together in the future to more rapidly advance our understanding and use of circadian principles for skeletal-muscle and metabolic health. Such knowledge can help to develop timed interventions that may aid to optimize treatment and prevention options for obesity and T2DM.O

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References

1. Miller BH, McDearmon EL, Panda S, et al. Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proc Natl Acad Sci U S A* 2007;104:3342-3347.
2. McCarthy JJ, Andrews JL, McDearmon EL, et al. Identification of the circadian transcriptome in adult mouse skeletal muscle. *Physiol Genomics* 2007;31:86-95.
3. Zhang R, Lahens NF, Ballance HI, Hughes ME, Hogenesch JB. A circadian gene expression atlas in mammals: implications for biology and medicine. *Proc Natl Acad Sci U S A* 2014;111:16219-16224.
4. Hodge BA, Wen Y, Riley LA, Stout DB, Herschman HR, Spencer MJ. The endogenous molecular clock orchestrates the temporal separation of substrate metabolism in skeletal muscle. *Skelet Muscle* 2015;5:15. doi:10.1186/s13395-015-0042-x
5. Janssen I, Heymsfield SB, Wang ZM, Ross R. Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. *J Appl Physiol (1985)* 2000;89:81-88.
6. Defronzo RA, Simonson D, Ferrannini E, Barrett E. Insulin resistance: a universal finding in diabetic states. *Bull Schweiz Akad Med Wiss* 1981;1981-1982:223-238.
7. Ferrannini E, Simonson DC, Katz LD, et al. The disposal of an oral glucose load in patients with non-insulin-dependent diabetes. *Metabolism* 1988;37:79-85.
8. Periasamy M, Herrera JL, Reis FCG. Skeletal muscle thermogenesis and its role in whole body energy metabolism. *Diabetes Metab J* 2017;41:327-336.
9. Zurlo F, Larson K, Bogardus C, Ravussin E. Skeletal muscle metabolism is a major determinant of resting energy expenditure. *J Clin Invest* 1990;86:1423-1427.
10. Zurlo F, Nemeth PM, Choksi RM, Sesodia S, Ravussin E. Whole-body energy metabolism and skeletal muscle biochemical characteristics. *Metabolism* 1994;43:481-486.
11. McCarthy JJ, Esser KA. Anabolic and catabolic pathways regulating skeletal muscle mass. *Curr Opin Clin Nutr Metab Care* 2010;13:230-235.
12. Nei M, Xu P, Glazko G. Estimation of divergence times from multiprotein sequences for a few mammalian species and several distantly related organisms. *Proc Natl Acad Sci U S A* 2001;98:2497-2502.
13. Acosta-Rodríguez VA, de Groot MHM, Rijo-Ferreira F, Green CB, Takahashi JS. Mice under caloric restriction self-impose a temporal restriction of food intake as revealed by an automated feeder system. *Cell Metab* 2017;26:267-277.e2.
14. Kohsaka A, Laposky AD, Ramsey KM, et al. High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab* 2007;6:414-421.
15. Campbell SS, Tobler I. Animal sleep: a review of sleep duration across phylogeny. *Neurosci Biobehav Rev* 1984;8:269-300.
16. Hughes ME, Abruzzi KC, Allada R, et al. Guidelines for genome-scale analysis of biological rhythms. *J Biol Rhythms* 2017;32:380-393.
17. Perrin L, Loizides-Mangold U, Chanon S, et al. Transcriptomic analyses reveal rhythmic and CLOCK-driven pathways in human skeletal muscle. *eLife* 2018;7:e34114. doi:10.7554/eLife.34114
18. van Moorsel D, Hansen J, Havekes B, et al. Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity. *Mol Metab* 2016;5:635-645.
19. Loizides-Mangold U, Perrin L, Vandereycken B, et al. Lipidomics reveals diurnal lipid oscillations in human skeletal muscle persisting in cellular myotubes cultured in vitro. *Proc Natl Acad Sci U S A* 2017;114:E8565-E8574.
20. Perrin L, Loizides-Mangold U, Skarupelova S, et al. Human skeletal myotubes display a cell-autonomous circadian clock implicated in basal myokine secretion. *Mol Metab* 2015;4:834-845.v
21. Hansen J, Timmers S, Moonen-Kornips E, et al. Synchronized human skeletal myotubes of lean, obese and type 2 diabetic patients maintain circadian oscillation of clock genes. *Sci Rep* 2016;6:35047. doi:10.1038/srep35047
22. Barnea M, Cohen-Yogev T, Chapnik N, Madar Z, Froy O. Effect of metformin and lipid emulsion on the circadian gene expression in muscle cells. *Int J Biochem Cell Biol* 2014;53:151-161.
23. Dyar KA, Ciciliot S, Wright LE, et al. Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. *Mol Metab* 2013;3:29-41.
24. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. *Physiol Rev* 2011;91:1447-1531.
25. Dyar KA, Ciciliot S, Tagliazucchi GM, et al. The calcineurin-NFAT pathway controls activity-dependent circadian gene expression in slow skeletal muscle. *Mol Metab* 2015;4:823-833.
26. Haldar SM, Jeyaraj D, Anand P, et al. Kruppel-like factor 15 regulates skeletal muscle lipid flux and exercise adaptation. *Proc Natl Acad Sci U S A* 2012;109:6739-6744.

27. Mansueto G, Armani A, Viscomi C, et al. Transcription factor EB controls metabolic flexibility during exercise. *Cell Metab* 2017;25:182-196.
28. Andrews JL, Zhang X, McCarthy JJ, et al. CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function. *Proc Natl Acad Sci U S A* 2010;107:19090-19095.
29. Zhang X, Patel SP, McCarthy JJ, Rabchevsky AG, Goldhamer DJ, Esser KA. A non-canonical E-box within the MyoD core enhancer is necessary for circadian expression in skeletal muscle. *Nucleic Acids Res* 2012;40:3419-3430.
30. Hodge BA, Zhang X, Gutierrez-Monreal MA, et al. MYOD1 functions as a clock amplifier as well as a critical co-factor for downstream circadian gene expression in muscle. *eLife* 2019;8:e43017. doi:10.7554/eLife.43017
31. Zitting K-M, Vujovic N, Yuan RK, et al. Human resting energy expenditure varies with circadian phase. *Curr Biol CB* 2018;28:3685-3690.e3.
32. Chtourou H, Souissi N. The effect of training at a specific time of day: a review. *J Strength Cond Res* 2012;26:1984-2005.
33. Facer-Childs E, Brandstaetter R. The impact of circadian phenotype and time since awakening on diurnal performance in athletes. *Curr Biol CB* 2015;25:518-522.
34. Saad A, Dalla Man C, Nandy DK, et al. Diurnal pattern to insulin secretion and insulin action in healthy individuals. *Diabetes* 2012;61:2691-2700.
35. Bae S-A, Fang MZ, Rustgi V, Zarbl H, Androulakis IP. At the interface of lifestyle, behavior, and circadian rhythms: metabolic implications. *Front Nutr* 2019;6:132. doi:10.3389/fnut.2019.00132
36. Bunger MK, Wilsbacher LD, Moran SM, et al. Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 2000;103:1009-1017.
37. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes Dev* 2006;20:1868-1873.
38. Rudic RD, McNamara P, Curtis A-M, et al. BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol* 2004;2:e377. doi:10.1371/journal.pbio.0020377
39. Harfmann BD, Schroder EA, Kachman MT, Hodge BA, Zhang X, Esser KA. Muscle-specific loss of Bmal1 leads to disrupted tissue glucose metabolism and systemic glucose homeostasis. *Skelet Muscle* 2016;6:12. doi:10.1186/s13395-016-0082-x
40. Vitaterna MH, King DP, Chang AM, et al. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* 1994;264:719-725.
41. King DP, Zhao Y, Sangoram AM, et al. Positional cloning of the mouse circadian clock gene. *Cell* 1997;89:641-653.
42. Chua EC-P, Shui G, Lee IT-G, et al. Extensive diversity in circadian regulation of plasma lipids and evidence for different circadian metabolic phenotypes in humans. *Proc Natl Acad Sci U S A* 2013;110:14468-14473.
43. Gan Y, Yang C, Tong X, et al. Shift work and diabetes mellitus: a meta-analysis of observational studies. *Occup Environ Med* 2015;72:72-78.
44. Vetter C, Dashti HS, Lane JM, et al. Night shift work, genetic risk, and type 2 diabetes in the UK biobank. *Diabetes Care* 2018;41:762-769.
45. Wefers J, van Moorsel D, Hansen J, et al. Circadian misalignment induces fatty acid metabolism gene profiles and compromises insulin sensitivity in human skeletal muscle. *Proc Natl Acad Sci U S A* 2018;115:7789-7794.
46. Cedernaes J, Schönke M, Westholm JO, et al. Acute sleep loss results in tissue-specific alterations in genome-wide DNA methylation state and metabolic fuel utilization in humans. *Sci Adv* 2018;4:eaar8590. doi:10.1126/sciadv.aar8590
47. Buxton OM, Cain SW, O'Connor SP, et al. Adverse metabolic consequences in humans of prolonged sleep restriction combined with circadian disruption. *Sci Transl Med* 2012;4:129ra43. doi:10.1126/scitranslmed.3003200
48. Ramsey KM, Yoshino J, Brace CS, et al. Circadian clock feedback cycle through NAMPT-mediated NAD^+ biosynthesis. *Science* 2009;324:651-654.
49. Benedict C, Shostak A, Lange T, et al. Diurnal rhythm of circulating nicotinamide phosphoribosyltransferase (Nampt/visfatin/PBEF): impact of sleep loss and relation to glucose metabolism. *J Clin Endocrinol Metab* 2012;97:E218-E222.
50. Sardon Puig L, Pillon NJ, Näslund E, Krook A, Zierath JR. Influence of obesity, weight loss, and free fatty acids on skeletal muscle clock gene expression. *Am J Physiol Endocrinol Metab* 2020;318:E1-E10.
51. Spiller S, Blüher M, Hoffmann R. Plasma levels of free fatty acids correlate with type 2 diabetes mellitus. *Diabetes Obes Metab* 2018;20:2661-2669.
52. Meex RCR, Schrauwen-Hinderling VB, Moonen-Kornips E, et al. Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. *Diabetes* 2010;59:572-579.
53. Bass J. Circadian topology of metabolism. *Nature* 2012;491:348-356.
54. Schroder EA, Esser KA. Circadian rhythms, skeletal muscle molecular clocks, and exercise. *Exerc Sport Sci Rev* 2013;41:224-229.
55. Schroeder AM, Truong D, Loh DH, Jordan MC, Roos KP, Colwell CS. Voluntary scheduled exercise alters diurnal rhythms of behaviour, physiology and gene expression in wild-type and vasoactive intestinal peptide-deficient mice. *J Physiol* 2012;590:6213-6226.
56. Zambon AC, McDearmon EL, Salomonis N, et al. Time- and exercise-dependent gene regulation in human skeletal muscle. *Genome Biol* 2003;4:R61. doi:10.1186/gb-2003-4-10-r61
57. Youngstedt SD, Elliott JA, Kripke DF. Human circadian phase-response curves for exercise. *J Physiol* 2019;597:2253-2268.
58. Miyazaki T, Hashimoto S, Masubuchi S, Honma S, Honma KI. Phase-advance shifts of human circadian pacemaker are accelerated by daytime physical exercise. *Am J Physiol Regul Integr Comp Physiol* 2001;281:R197-R205.
59. Eastman CI, Hoese EK, Youngstedt SD, Liu L. Phase-shifting human circadian rhythms with exercise during the night shift. *Physiol Behav* 1995;58:1287-1291.
60. Van Reeth O, Sturis J, Byrne MM, et al. Nocturnal exercise phase delays circadian rhythms of melatonin and thyrotropin secretion in normal men. *Am J Physiol* 1994;266:E964-E974.
61. Baehr EK, Eastman CI, Revelle W, Olson SHL, Wolfe LF, Zee PC. Circadian phase-shifting effects of nocturnal exercise in older compared with young adults. *Am J Physiol Regul Integr Comp Physiol* 2003;284:R1542-R1550.
62. Sato S, Basse AL, Schönke M, et al. Time of exercise specifies the impact on muscle metabolic pathways and systemic energy homeostasis. *Cell Metab* 2019;30:92-110.e4.
63. Ezagouri S, Zwighaft Z, Sobel J, et al. Physiological and molecular dissection of daily variance in exercise capacity. *Cell Metab* 2019;30:78-91.e4.
64. Jordan SD, Kriebs A, Vaughan M, et al. CRY1/2 selectively repress PPARδ and limit exercise capacity. *Cell Metab* 2017;26:243-255.e6.
65. Lassiter DG, Sjögren RJO, Gabriel BM, Krook A, Zierath JR. AMPK activation negatively regulates GDAP1, which influences metabolic processes and circadian gene expression in skeletal muscle. *Mol Metab* 2018;16:12-23.
66. Peek CB, Levine DC, Cedernaes J, et al. Circadian clock interaction with HIF1α mediates oxygenic metabolism and anaerobic glycolysis in skeletal muscle. *Cell Metab* 2017;25:86-92.
67. Savikj M, Gabriel BM, Alm PS, et al. Afternoon exercise is more efficacious than morning exercise at improving blood glucose levels in individuals with type 2 diabetes: a randomised crossover trial. *Diabetologia* 2019;62:233-237.